RRP SEQUENCES AND KNOCKOUT MICE AND USES THEREOF

REFERENCE TO RELATED APPLICATIONS

[0001] This application is a Continuation In Part of US utility patent application 09/908,419, entitled "Human RRP Sequences and Methods of Use" filed 7/18/2001, which claims priority to U.S. provisional patent applications 60/219,289, filed 7/19/2000, 60/277,487, filed 3/21/2001, 60/277,471, filed 3/21/2001, and 60/304,863, filed 7/12/2001.

[0002] Priority is also claimed to U.S. provisional patent applications 60/296,076 filed 6/5/2001, 60/305,017 filed 7/12/2001, 60/328,605 filed 10/10/2001, and 60/328,491 filed 10/10/2001. The contents of the prior applications are hereby incorporated in their entirety.

BACKGROUND OF THE INVENTION

[0003] Signal transduction pathways are made up of growth factors, their receptors, upstream regulators of the growth factors, and downstream intracellular kinase networks. These pathways regulate many cellular processes, including proliferation, and appear to play a key role in oncogenesis.

[0004] The epidermal growth factor receptor (EGFR) and its pathway members are among the most widely explored signaling pathways. Signaling through this pathway elicits diverse biological responses whose manifestations can include mitogenesis or apoptosis, enhanced cell motility, protein secretion, and differentiation or dedifferentiation. Up-regulated EGFR signaling has been implicated in organ morphogenesis, maintenance and repair, and is correlated with invasion and metastasis of many types of tumors. Upregulated EGF and EGFR in various tumor types leads to increased expression of p21/Waf1/Cip1, leading to an arrest in the G1 phase of the cell cycle (Reddy KB et al., (1999) Int. J. Cancer 15:301-306).

[0005] Signal transduction pathways, such as the EGFR pathway, are evolutionarily conserved among species as distant as the worm *Caenorhabditis elegans*, the fruit fly

Drosophila melanogaster, and vertebrates (Duffy JB, and Perrimon N, Curr. Opin. Cell Biol. (1996) 8:231-238). In fact, ligands for the *Drosophila* EGFR (DER), known as Spitz (Rutledge B, et al, Genes Dev. (1992) 6:1503-1517) and Gurken (Neuman-Silberberg FS, and Schupbach T, Cell (1993) 75:165-174), are both similar to TGFα (transforming growth factor alpha), the ligand for the vertebrate EGFR (Massague J, J Biol Chem. (1990) 265:21393-21396). The rhomboid gene, which encodes a transmembrane protein, is another upstream member of this pathway (Bier E., et al., Genes Dev. (1990) 4:190-203). In *Drosophila*, rhomboid protein is a transmembrane serine protease, cleaves Spitz, and transforms Spitz from a membrane-bound to a secreted form, and thus triggers and upregulates the DER signaling pathway (Wasserman JD et al, Genes Devel (2000) 14:1651-1663; Lee JR, et al., (2001) Cell 107:161-171; Urban S, et al., (2001) Cell 107: 173-182). DNA sequences related to rhomboid have been identified in *C. elegans* (Wasserman JD, and Freeman M, Trends Cell Biol (1997) 7:431-436), and in mammals (Pascall JC, and Brown KD, FEBS letters (1998) 429:337-340; human: GI#3287191 and GI#7020534, among others; and rat: GI#3297936;), suggesting that rhomboid function may be evolutionarily conserved. Modulation of EGF-receptor related activity by synthetic peptides or humanized monoclonal antibodies inhibit tumor growth (Baselga J, et al., (1998) Cancer Research 58:2825-2831; Park B-W, et al., (2000) Nature Biotechnology 18:194-197).

[0006] The p53 gene is mutated in over 50 different types of human cancers, including familial and spontaneous cancers, and is believed to be the most commonly mutated gene in human cancer (Zambetti and Levine, FASEB (1993) 7:855-865; Hollstein, *et al.*, Nucleic Acids Res. (1994) 22:3551-3555). Greater than 90% of mutations in the p53 gene are missense mutations that alter a single amino acid that inactivates p53 function. Aberrant forms of human p53 are associated with poor prognosis, more aggressive tumors, metastasis, and short survival rates (Mitsudomi et al., Clin Cancer Res 2000 Oct; 6(10):4055-63; Koshland, Science (1993) 262:1953).

[0007] The human p53 protein normally functions as a central integrator of signals including DNA damage, hypoxia, nucleotide deprivation, and oncogene activation (Prives, Cell (1998) 95:5-8). In response to these signals, p53 protein levels are

greatly increased with the result that the accumulated p53 activates cell cycle arrest (through activation of p21/Waf1/Cip1) or apoptosis depending on the nature and strength of these signals. Indeed, multiple lines of experimental evidence have pointed to a key role for p53 as a tumor suppressor (Levine, Cell (1997) 88:323-331). For example, homozygous p53 "knockout" mice are developmentally normal but exhibit nearly 100% incidence of neoplasia in the first year of life (Donehower *et al.*, Nature (1992) 356:215-221).

[0008] The biochemical mechanisms and pathways through which p53 functions in normal and cancerous cells are not fully understood, but one clearly important aspect of p53 function is its activity as a gene-specific transcriptional activator. Among the genes with known p53-response elements are several with well-characterized roles in either regulation of the cell cycle or apoptosis, including GADD45, p21/Waf1/Cip1, cyclin G, Bax, IGF-BP3, and MDM2 (Levine, Cell (1997) 88:323-331).

[0009] Modulating signal transduction pathway activity involved in tumor growth and development is essential in understanding the development of many cancers, and eventually, for the treatment of cancer. The ability to manipulate the genomes of model organisms such as *Drosophila* provides a powerful means to analyze signal transduction pathwasy that, due to significant evolutionary conservation, have direct relevance to more complex vertebrate organisms. Due to a high level of gene and pathway conservation, the strong similarity of cellular processes, and the functional conservation of genes between these model organisms and mammals, identification of the involvement of novel genes in particular pathways and their functions in such model organisms can directly contribute to the understanding of the correlative pathways and methods of modulating them in mammals (see, for example, Mechler BM et al., 1985 EMBO J 4:1551-1557; Gateff E. 1982 Adv. Cancer Res. 37: 33-74; Watson KL., et al., 1994 J Cell Sci. 18: 19-33; Miklos GL, and Rubin GM. 1996 Cell 86:521-529; Wassarman DA, et al., 1995 Curr Opin Gen Dev 5: 44-50; and Booth DR. 1999 Cancer Metastasis Rev. 18: 261-284). For example, a genetic screen can be carried out in an invertebrate model organism having underexpression (e.g. knockout) or overexpression of a gene (referred to as a "genetic entry point") that yields a visible phenotype. Additional genes are mutated in a random or targeted

manner. When a gene mutation changes the original phenotype caused by the mutation in the genetic entry, the gene is identified as a "modifier" involved in the same or overlapping pathway as the genetic entry point. When the genetic entry point is an ortholog of a human gene implicated in a disease pathway, such as p53 or p21, modifier genes can be identified that may be attractive candidate targets for novel therapeutics.

[0010] All references cited herein, including sequence information in referenced Genbank identifier numbers and website references, are incorporated herein in their entireties.

SUMMARY OF THE INVENTION

[0011] We have discovered genes that modify the p53 or p21 pathway in *Drosophila*, and identified their mammalian orthologs, hereinafter referred to as Rhomboid Related Proteins (RRP), and more specifically, RRP1 – RRP8, and mouse RRP1 (mRRP1). The invention provides isolated nucleic acid molecules that comprise nucleic acid sequences encoding RRP protein as well as fragments and derivatives thereof. Vectors and host cells comprising the RRP nucleic acid molecules are also described.

[0012] The invention provides methods for utilizing these p53 or p21 modifier genes and polypeptides to identify RRP modulating agents, which are candidate therapeutic agents that can be used in the treatment of disorders associated with defective p53 or p21 function.

[0013] In one embodiment, candidate p53 or p21 modulating agents are tested with an assay system comprising a RRP polypeptide or nucleic acid. Candidate agents that produce a change in the activity of the assay system relative to controls are identified as candidate p53 or p21 modulating agents. The assay system may be cell-based or cell-free. Candidate modulating agents include small molecule modulators, antibodies, and nucleic acid modulators. In one specific embodiment, a small molecule modulator is identified using a protease assay. In specific embodiments, the screening assay system is selected from an apoptosis assay, a cell proliferation assay, an angiogenesis assay, and a hypoxic induction assay.

[0014] In another embodiment, candidate p53 or p21 pathway modulating agents are further tested using a second assay system that detects changes in the p53 or p21 pathway, such as angiogenic, apoptotic, or cell proliferation changes produced by the originally identified candidate agent or an agent derived from the original agent. The second assay system may use cultured cells or non-human animals. In specific embodiments, the secondary assay system uses non-human animals, including animals predetermined to have a disease or disorder implicating the p53 or p21 pathway, such as an angiogenic, apoptotic, or cell proliferation disorder (e.g. cancer).

[0015] The invention further provides methods for modulating the p53 or p21 pathway in a mammalian cell by contacting the mammalian cell with an agent that specifically binds a RRP polypeptide or nucleic acid. The agent may be a small molecule modulator, a nucleic acid modulator, or an antibody and may be administered to a mammalian animal predetermined to have a pathology associated the p53 or p21 pathway.

[0016] Modulating agents identified using the methods of the invention can be used to specifically inhibit growth of tumor cells that overexpress an RRP protein.

[0017] The invention also provides transgenic knockout mice harboring disrupted RRP genes. The disruption may be heterozygous, leading to decreased expression of RRP, or homozygous, leading to lack of expression of the RRP gene. Cells from the mice as well as cells harboring disrupted RRP genes are also provided. Methods of producing antibody to RRP using the mice of the invention are also provided.

[0018] Targeting vectors to produce transgenic knockout mice are also provided. Preferably, a targeting vector is provided that allows sequential deletion of vector sequences from the same cell in the generation of the knockout mice.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] FIG.1 depicts the targeting vector, pEasyfloxFRTGK12 mRRP1.

[0020] FIG.2 shows the mRRP1 mouse targeting vector, the mRRP1 wild type (WT) genomic region and the locations to which the vector is targeted, and the mRRP1 target allele after homologous recombination (HR). Locations of the 5' external probe, the internal probe, and the 3' external probe are underlined. The expected

sizes of the genomic fragments after digestion with the indicated restriction enzymes and Southern hybridization are also indicated.

[0021] FIG.3 depicts the target allele after HR and after Cre induced deletion. The expected sizes of the genomic fragments after digestion with restriction enzymes and Southern hybridization are indicated for the target allele in each of the following states: WT, HR, and Cre deleted.FIG.4 depicts the target allele after HR, after FLP induced deletion, and after Cre induced deletion. The expected sizes of the genomic fragments after digestion with the indicated restriction enzymes and Southern hybridization are indicated for the target allele in each of the following states: WT, HR, FLP deleted, and Cre deleted.

DETAILED DESCRIPTION OF THE INVENTION

[0023] Genetic screens were designed to identify modifiers of the p53 or p21 pathways in *Drosophila*. Genetic modifier screens were carried out in which p53 (Ollmann M, et al., Cell 2000 101: 91-101) or p21 (Bourne HR, et al., Nature (1990) 348(6297):125-132; Marshall CJ, Trends Genet (1991) 7(3):91-95) were overexpressed. *Drosophila* rhomboid genes were identified as modifiers of the p53 or p21 pathways. Accordingly, vertebrate orthologs of these modifiers, hereinafter referred to as RRP genes (i.e., nucleic acids and polypeptides), are attractive drug targets for the treatment of pathologies associated with a defective p53 or p21 signaling pathways, such as cancer. Further, gene targeting in mice is an ideal method to investigate the function of a distinct protein in wild type and disease states. In order to study the RRP1 function in mammals we generated the genomic sequence of the RRP1 region in mice, deduced its cDNA and protein sequence, and then produced targeted RRP1 knockout (KO) mice.

[0024] In vitro and in vivo methods of assessing RRP function as provided herein, and modulation of the RRP or their respective binding partners is useful for understanding the association of the p53 or p21 pathways and their members in normal and disease conditions and for developing diagnostics and therapeutic modalities for p53 or p21 related pathologies. RRP-modulating agents that act by inhibiting or enhancing RRP expression, directly or indirectly, for example, by affecting an RRP function such as

enzymatic (e.g., catalytic) or binding activity, can be identified using methods provided herein. RRP-modulating agents include RRP related proteins (e.g. dominant negative mutants, and biotherapeutics); RRP-specific antibodies; RRP-specific antisense oligomers; and chemical agents that specifically bind RRP or compete with RRP binding target. The invention provides methods of identifying and making RRP modulating agents, and their use in diagnosis, therapy and pharmaceutical development.

[0025] Preferred RRP-modulating agents specifically bind to RRP polypeptides and enhance or inhibit RRP function. Other preferred RRP-modulating agents are antisense oligomers and RNAi that repress RRP gene expression or product activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA). RRP-specific modulating agents may be evaluated by any convenient *in vitro* or *in vivo* assay for molecular interaction with an RRP polypeptide or nucleic acid.

[0026] The method of this invention is useful in the therapy of malignant or benign tumors of mammals that overexpress RRP gene products.

Nucleic acids and polypeptides of the invention

[0027] Sequences related to RRP nucleic acids (RRP1: SEQ ID NO:1, RRP2: SEQ ID NO:3, RRP3: SEQID NO:5, RRP4: SEQ ID NO:7, RRP5: SEQ ID NO:9, RRP6: SEQ ID NO:11, RRP7: SEQ ID NO:13, and RRP8: SEQ ID NO:15) and polypeptides (RRP1: SEQ ID NO:2, RRP2: SEQ ID NO: 4, RRP3: SEQID NO:6, RRP4: SEQ ID NO:8, RRP5: SEQ ID NO:10, RRP6: SEQ ID NO:12, RRP7: SEQ ID NO:14, and RRP8: SEQ ID NO:16) are available in the public databases (for RRP1: cDNA: GI#3287190, SEQ ID NO:18; proteins GI#3287191, SEQ ID NO:36; for RRP2: cDNAs: GI#s: 12762689 (SEQ ID NO:19), 12096415 (SEQ ID NO:20), 6657080 (SEQ ID NO:21), 7947756 (SEQ ID NO:22), 3117010 (SEQ ID NO:23), 9339870 (SEQ ID NO:24), 7152496 (SEQ ID NO:25), 11317512 (SEQ ID NO:26), 11317513 (SEQ ID NO:27), and 11317511 (SEQ ID NO:28); proteins: GI#s:8923409 (SEQ ID NO:37), 12719522 (SEQ ID NO:38), and 11421817 (SEQ ID NO:39); for RRP3: cDNA: GI#10199673 (SEQ ID NO:29) and GI#2003992 (SEQ ID NO:30); for RRP4:

cDNA GI#11066249 (SEQ ID NO:31) and protein GI#11066250 (SEQ ID NO:40); for RRP5: cDNA GI#11967982 (SEQ ID NO:32) and protein GI#11967983 (SEQ ID NO:41); for RRP6: cDNA GI#10438685 (SEQ ID NO:33) and protein GI#10438686 (SEQ ID NO:42); for RRP7: cDNA GI#10190733 (SEQ ID NO:34) and protein GI#10190734 (SEQ ID NO:43); and for RRP8: cDNA GI#11072100 (SEQ ID NO:35) and protein GI#11072101 (SEQ ID NO:44)). Sequences of human and rat RRP1 were used to deduce the mouse RRP1 (mRRP1) cDNA (SEQ ID NO:45), polypeptide (SEQ ID NO:46), and genomic (SEQ ID NO:47) sequences, as described in Example VII. The mRRP1 cDNA sequence shares 69% identity with human RRP1 and 88% identity with rat partial RRP1 for nucleotides 884-1340 of the mouse RRP1. The mRRP1 protein shares 80% identity with human RRP1 and 88% identity with rat partial RRP1 for amino acids 297-448 of mRRP1.

[0028] RRPs are a family of integral membrane proteins that contain five or more transmembrane domains and three strongly conserved histidine residues in the putative transmembrane regions. In a preferred embodiment, the invention provides RRP proteins which comprise or consist of an amino acid sequence of SEQ ID NOs:4, 6, or 46, or fragments or derivatives thereof.

[0029] The term "RRP polypeptide" refers to a full-length RRP protein or a functionally active fragment or derivative thereof. A "functionally active" RRP fragment or derivative exhibits one or more functional activities associated with a full-length, wild-type RRP protein, such as antigenic or immunogenic activity, enzymatic activity, ability to bind natural cellular substrates, etc. The functional activity of RRP proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey) and as further discussed below. For purposes herein, functionally active fragments also include those fragments that comprise one or more structural domains of an RRP, such as a protease or rhomboid domain or a binding domain. Catalytic and other domains can be identified using the PFAM program (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2; http://pfam.wustl.edu). Methods for obtaining RRP polypeptides are also further described below. Preferred fragments are functionally active, domain-containing

fragments sharing at least 80% sequence identity or similarity, preferably at least 85%, more preferably at least 90%, and most preferably at least 95% sequence identity or similarity with a contiguous stretch of at least 25 amino acids, preferably at least 50 amino acids, more preferably at least 100 amino acids, and in some cases, the entire length of any one of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, and 46. In further preferred embodiments, the fragment comprises the entire rhomboid domain (PFAM 01694).

[0030] RRP protein derivatives typically share a certain degree of sequence identity or sequence similarity with SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, or 46 or a fragment thereof. RRP derivatives can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, a cloned RRP gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) (Wells *et al.*, Philos. Trans. R. Soc. London SerA (1986) 317:415), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*, and expressed to produce the desired derivative. Alternatively, an RRP gene can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or to form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. A variety of mutagenesis techniques are known in the art such as chemical mutagenesis, *in vitro* site-directed mutagenesis (Carter *et al.*, Nucl. Acids Res. (1986) 13:4331), use of TAB® linkers (available from Pharmacia and Upjohn, Kalamazoo, MI), *etc.*

[0031] At the protein level, manipulations include post translational modification, *e.g.* glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, *etc.* Any of numerous chemical modifications may be carried out by known technique (*e.g.* specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, *etc.*). Derivative proteins can also be chemically synthesized by use of a peptide

synthesizer, for example to introduce nonclassical amino acids or chemical amino acid analogs as substitutions or additions into the RRP protein sequence.

[0032] Chimeric or fusion proteins can be made comprising an RRP protein or fragment thereof (preferably comprising one or more structural or functional domains of the RRP protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. Chimeric proteins can be produced by any known method, including: recombinant expression of a nucleic acid encoding the protein (comprising a RRP-coding sequence joined in-frame to a coding sequence for a different protein); ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other in the proper coding frame, and expressing the chimeric product; and protein synthetic techniques, *e.g.* by use of a peptide synthesizer.

[0033] The subject RRP polypeptides also encompass minor deletion mutants, including N-, and/or C-terminal truncations. Such deletion mutants are readily screened for RRP competitive or dominant negative activity.

[0034] The term "RRP nucleic acid" refers to a DNA or RNA molecule that encodes a RRP polypeptide. In preferred embodiments, the nucleic acid encodes a polypeptide selected from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, and 46. In some embodiments, the nucleic acid comprises a sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 7, 9, 11, 13, 15, and 45. In a specific embodiment, the invention provides an isolated nucleic acid which encodes a human RRP3 as shown in SEQ ID NO:5, and also an isolated nucleic acid that encodes a mouse RRP (mRRP1) as shown in SEQ ID NO:45.

[0035] The invention includes a fragment of a nucleic acid, such as a fragment that encodes a binding domain of one of the full-length sequences of the invention. Fragments of an RRP nucleic acid sequence can be used for a variety of purposes. As an example, interfering RNA (RNAi) fragments, particularly double-stranded (ds) RNAi, can be used to generate loss-of-function phenotypes; which can, in turn, be used, among other uses, to determine gene function. Certain "antisense" fragments, i.e. that are reverse complements of portions of the coding and/or untranslated regions (e.g. 5' UTR) have utility in inhibiting the function of RRP proteins. The fragments

are of length sufficient to specifically hybridize with the corresponding RRP sequence. The fragments consist of or comprise at least 12, preferably at least 24, more preferably at least 36, and more preferably at least 96 contiguous nucleotides of RRP. When the fragments are flanked by other nucleic acid sequences, the total length of the combined nucleic acid sequence is less than 15 kb, preferably less than 10 kb or less than 5kb, more preferably less than 2 kb, and in some cases, preferably less than 500 bases.

[0036] In other specific embodiments, preferred fragments of SEQ ID NO:5 encode extracellular or intracellular domains which are located at approximately nucleotides 248-598, 665-796, 862-870, 934-943, 1006-1138, 1201-1225, and 1289-1336. Additional preferred fragments of SEQ ID NO:45 encode extracellular or intracellular domains which are located at approximately nucleotides 1-714, 774-912, 972-984, 1044-1089, 1149-1212, 1272-1305, 1365-1408. Preferred fragments may also include a binding domain or an RRP motif (e.g. PFAM 01694). These domains may be useful to locate the function and/or binding partners of a protein. For example, a nucleic acid that encodes an extracellular or intracellular domain of a protein may be used to screen for binding partners related to the protein.

[0037] The subject nucleic acid sequences may consist solely of the RRP nucleic acid or fragments thereof. Alternatively, the subject nucleic acid sequences and fragments thereof may be joined to other components such as labels, peptides, agents that facilitate transport across cell membranes, hybridization-triggered cleavage agents or intercalating agents. The subject nucleic acid sequences and fragments thereof may also be joined to other nucleic acid sequences (i.e. they may comprise part of larger sequences) and are of synthetic/non-natural sequences and/or are isolated and/or are purified, i.e. unaccompanied by at least some of the material with which it is associated in its natural state. Preferably, the isolated nucleic acids constitute at least about 0.5%, and more preferably at least about 5% by weight of the total nucleic acid present in a given fraction, and are preferably recombinant, meaning that they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome.

[0038] The subject nucleic acids find a wide variety of applications including use as translatable transcripts, hybridization probes, PCR primers, diagnostic nucleic acids, etc.; use in detecting the presence of RRP genes and gene transcripts and in detecting or amplifying nucleic acids encoding additional RRP homologs and structural analogs. In diagnosis, RRP hybridization probes find use in identifying wild-type and mutant RRP alleles in clinical and laboratory samples. Mutant alleles are used to generate allele-specific oligonucleotide (ASO) probes for high-throughput clinical diagnoses. In therapy, therapeutic RRP nucleic acids are used to modulate cellular expression or intracellular concentration or availability of active RRP. In a preferred embodiment, the mouse RRP1 sequence is used to produce a targeting vector for production of mice that are deficient in RRP1 in a heterozygous or homozygous (i.e., knockout) manner.

[0039] In one preferred embodiment, the derivative nucleic acid encodes a polypeptide comprising a RRP3 amino acid sequence of SEQ ID NO:6, an mRRP1 amino acid sequence of SEQ ID NO:46, or a fragment or derivative thereof. A derivative RRP3 nucleic acid sequence, or fragment thereof, may comprise 100% sequence identity with SEQ ID NO:5 or 45, but be a derivative thereof in the sense that it has one or more modifications at the base or sugar moiety, or phosphate backbone. Examples of modifications are well known in the art (Bailey, Ullmann's Encyclopedia of Industrial Chemistry (1998), 6th ed. Wiley and Sons). Such derivatives may be used to provide modified stability or any other desired property.

[0040] Preferably, the RRP polypeptide nucleic acid, fragment, ortholog, or derivative thereof has at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably 90%, and most preferably at least 95% sequence identity with RRP. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. As used herein, "percent (%) sequence identity" with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent

sequence identity, as generated by the program WU-BLAST-2.0a19 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410; http://blast.wustl.edu/blast/README.html) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

[0041] A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

[0042] Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, Advances in Applied Mathematics 2:482-489; database: European Bioinformatics Institute wwwz.ebi.ac.uk/bic.sub.-- sw/; Smith and Waterman, 1981, J. of Molec.Biol., 147:195-197; Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.; W.R. Pearson, 1991, Genomics 11:635-650). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff (Dayhoff: Atlas of Protein Sequences and Structure, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA), and normalized by Gribskov (Gribskov 1986 Nucl. Acids Res. 14(6):6745-6763). The Smith-

Waterman algorithm is used to search databases for sequences similar to a query sequence. Smith-Waterman uses dynamic programming to determine how an optimal alignment between the query sequence and a database sequence can be produced. This alignment is obtained by determining what transformations the query sequence would need to undergo to match the database sequence. Transformations include substituting one character for another and inserting or deleting a string of characters. A score is assigned for each character-to-character comparison--positive scores for exact matches and some substitutions, negative scores for other substitutions and insertions/deletions. The first character in an insertion or deletion gap is scored with a gap open penalty and subsequent characters are scored with a gap extension penalty. Scores are obtained from statistically-derived scoring matrices. The combination of transformations that results in the highest score is used to generate an alignment between the query sequence and database sequence. Smith-Waterman algorithm may be employed where default parameters are used for scoring (for example, gap open penalty of 12, gap extension penalty of two). From the data generated the "Match" value reflects "sequence identity."

[0043] Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of SEQ ID NOs:1, 3, 5, 7, 9, 1, 13, 15, or 45. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are set out in readily available procedure texts (*e.g.*, Current Protocol in Molecular Biology, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of any one of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, or 45 under stringent hybridization conditions that comprise: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 μg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C in a solution containing 6X SSC, 1X

Denhardt's solution, 100 μg/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1h in a solution containing 0.2X SSC and 0.1% SDS (sodium dodecyl sulfate).

[0044] In other embodiments, moderately stringent hybridization conditions are used that comprise: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA; hybridization for 18-20h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

[0045] Alternatively, low stringency conditions can be used that comprise: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

Isolation, Production, Expression, and Mis-expression of RRP Nucleic Acids and Polypeptides

[0046] RRP nucleic acids and polypeptides, useful for identifying and testing agents that modulate RRP function and for other applications related to the involvement of RRP in the p53 or p21 pathways. RRP nucleic acids and derivatives and orthologs thereof may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase chain reaction (PCR) are well known in the art. In general, the particular use for the protein will dictate the particulars of expression, production, and purification methods. For instance, production of proteins for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*,

generation of fusion proteins). Overexpression of an RRP protein for assays used to assess RRP function, such as involvement in cell cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and purification of proteins are well known in the art; any suitable means therefore may be used (e.g., Higgins SJ and Hames BD (eds.) Protein Expression: A Practical Approach, Oxford University Press Inc., New York 1999; Stanbury PF et al., Principles of Fermentation Technology, 2nd edition, Elsevier Science, New York, 1995; Doonan S (ed.) Protein Purification Protocols, Humana Press, New Jersey, 1996; Coligan JE et al, Current Protocols in Protein Science (eds.), 1999, John Wiley & Sons, New York; U.S. Pat. No. 6,165,992). In particular embodiments, recombinant RRP is expressed in a cell line known to have defective p53 or p21 function (e.g. for p53: SAOS-2 osteoblasts, H1299 lung cancer cells, C33A and HT3 cervical cancer cells, HT-29 and DLD-1 colon cancer cells, among others, and for p21: HCT116 colon cancer cells, among others, available from American Type Culture Collection (ATCC), Manassas, VA). The recombinant cells are used in cell-based screening assay systems of the invention, as described further below.

[0047] The nucleotide sequence encoding an RRP polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals, including promoter/enhancer element, can derive from the native RRP gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may be utilized, such as mammalian cell systems infected with virus (*e.g.* vaccinia virus, adenovirus, *etc.*); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, plasmid, or cosmid DNA. A host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

[0048] To detect expression of the RRP gene product, the expression vector can comprise a promoter operably linked to an RRP gene nucleic acid, one or more origins of replication, and, one or more selectable markers (*e.g.* thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the RRP gene product based on the

physical or functional properties of the RRP protein in *in vitro* assay systems (*e.g.* immunoassays).

[0049] The RRP protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product (i.e. it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the chimeric product. A chimeric product may also be made by protein synthetic techniques, *e.g.* by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

[0050] Once a recombinant cell that expresses the RRP gene sequence is identified, the gene product can be isolated and purified using standard methods (*e.g.* ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility; electrophoresis, cite purification reference). Alternatively, native RRP proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification). Once a protein is obtained, it may be quantified and its activity measured by appropriate methods, such as immunoassay, bioassay, or other measurements of physical properties, such as crystallography.

[0051] The methods of this invention may also use cells that have been engineered for altered expression (mis-expression) of RRP or other genes associated with the p53 or p21 pathway. As used herein, mis-expression encompasses ectopic expression, over-expression, under-expression, and non-expression (*e.g.* by gene knock-out or blocking expression that would otherwise normally occur).

Genetically modified animals

[0052] Animal models that have been genetically modified to alter RRP expression may be used in *in vivo* assays to test for activity of a candidate p53 or p21 modulating agent, or to further assess the role of RRP in a p53 or p21 pathway process such as apoptosis or cell proliferation. Preferably, the altered RRP expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal RRP

expression. The genetically modified animal may additionally have altered p53 or p21 expression (e.g. p53 or p21 knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice), cows, horses, goats, sheep, pigs, dogs and cats. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, i.e. mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4:761-763.) or stably integrated into its germ line DNA (i.e., in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

[0053] Methods of making transgenic animals are well-known in the art (for transgenic mice see Brinster et al., Proc. Nat. Acad. Sci. USA 82: 4438-4442 (1985), U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al., U.S. Pat. No. 6,127,598, by German *et al.*, and Hogan, B., Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for particle bombardment see U.S. Pat. No., 4,945,050, by Sandford *et al.*; for transgenic *Drosophila* see Rubin and Spradling, Science (1982) 218:348-53 and U.S. Pat. No. 4,670,388; for transgenic insects see Berghammer A.J. *et al.*, A Universal Marker for Transgenic Insects (1999) Nature 402:370-371; for transgenic Zebrafish see Lin S., Transgenic Zebrafish, Methods Mol Biol. (2000);136:375-3830); for microinjection procedures for fish, amphibian eggs and birds see Houdebine and Chourrout, Experientia (1991) 47:897-905; for transgenic rats see Hammer *et al.*, Cell (1990) 63:1099-1112; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, e.g., Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E. J. Robertson, ed., IRL Press (1987)). Clones of the nonhuman transgenic animals can be produced according to available methods (see Wilmut, I. *et al.* (1997) Nature 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

[0054] In one embodiment, the transgenic animal is a "knock-out" animal having a heterozygous or homozygous alteration in the sequence of an endogenous RRP gene that results in a decrease of RRP function, preferably such that RRP expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a mouse RRP gene is used to construct a homologous recombination vector suitable for altering an endogenous RRP gene in the mouse genome as shown in Example VIII. Detailed methodologies for homologous recombination in mice are available (see Capecchi, Science (1989) 244:1288-1292; Joyner *et al.*, Nature (1989) 338:153-156). Procedures for the production of non-rodent transgenic mammals and other animals are also available (Houdebine and Chourrout, *supra*; Pursel *et al.*, Science (1989) 244:1281-1288; Simms *et al.*, Bio/Technology (1988) 6:179-183). In a preferred embodiment, knock-out animals, such as mice harboring a knockout of a specific gene, may be used to produce antibodies against the human counterpart of the gene that has been knocked out (Claesson MH et al. (1994) Scand J Immunol 40:257-264; Declerck PJ, et al., (1995) J Biol Chem 270:8397-8400).

[0055] In another embodiment, the transgenic animal is a "knock-in" animal having an alteration in its genome that results in altered expression (e.g., increased (including ectopic) or decreased expression) of the RRP gene, e.g., by introduction of additional copies of RRP, or by operatively inserting a regulatory sequence that provides for altered expression of an endogenous copy of the RRP gene. Such regulatory sequences include inducible, tissue-specific, and constitutive promoters and enhancer elements. The knock-in can be homozygous or heterozygous.

[0056] Transgenic nonhuman animals can also be produced that contain selected systems allowing for regulated expression of the transgene. One example of such a system that may be produced is the cre/loxP recombinase system of bacteriophage P1 (Lakso *et al.*, PNAS (1992) 89:6232-6236; U.S. Pat. No. 4,959,317). If a cre/loxP

recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase. Another example of a recombinase system is the FLP recombinase system of Saccharomyces cerevisiae (O'Gorman et al. (1991) Science 251:1351-1355; U.S. Pat. No. 5,654,182). In a preferred embodiment, both Cre-LoxP and Flp-Frt are used in the same system to regulate expression of the transgene, and for sequential deletion of vector sequences in the same cell, such as shown in Example VIII.

[0057] The genetically modified animals can be used in genetic studies to further elucidate the p53 or p21 pathway, as animal models of disease and disorders implicating defective p53 or p21 function, and for *in vivo* testing of candidate therapeutic agents, such as those identified in the screens described below. Gene targeting in mice is an ideal method to investigate the function of a distinct protein in wild type and disease states. Further, animal models deficient in rhomboid RRP sequence and function are desirable tools for modulating the EGFR signaling pathway, for testing the effect of candidate compounds against RRP, and for production of antibodies against human RRP, among others. The candidate therapeutic agents are administered to a genetically modified animal having altered RRP function and phenotypic changes are compared with appropriate control animals such as genetically modified animals that receive placebo treatment, and/or animals with unaltered RRP expression that receive candidate therapeutic agent.

[0058] In additional to the above-described genetically modified animals having altered RRP function, animal models having defective p53 or p21 function (and otherwise normal RRP function), can be used in the methods of the present invention. For example, a p53 or p21 knockout mouse can be used to assess, *in vivo*, the activity of a candidate p53 or p21 modulatory agent identified in one of the *in vitro* assays described below. p53 or p21 knockout mice are described in the literature (p53: Jacks et al., Nature 2001;410:1111-1116, 1043-1044; Donehower *et al.*, supra; p21:Umanoff H, et al., Proc Natl Acad Sci U S A 1995 Feb 28;92(5):1709-13).

Modulating Agents

[0059] The invention provides methods to identify agents that interact with and/or modulate the function of RRP and/or the p53 or p21 pathway. Such agents are useful in a variety of diagnostic and therapeutic applications associated with the p53 or p21 pathways, as well as in further analysis of the RRP protein and its contribution to the p53 or p21 pathways. Accordingly, the invention also provides methods for modulating the p53 or p21 pathway comprising the step of specifically modulating RRP activity by administering an RRP-interacting or -modulating agent.

[0060] In a preferred embodiment, RRP-modulating agents inhibit or enhance RRP activity or otherwise affect normal RRP function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In a further preferred embodiment, the candidate p53 or p21 pathway- modulating agent specifically modulates the function of the RRP. The phrases "specific modulating agent", "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the RRP polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the RRP. The term also encompasses modulating agents that alter the interaction of the RRP with a binding partner or substrate (e.g. by binding to a binding partner of an RRP, or to a protein/binding partner complex, and inhibiting function).

[0061] Preferred RRP-modulating agents include small molecule compounds; RRP-interacting proteins, including antibodies and other biotherapeutics; antisense and RNA inhibitors. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19th edition.

Small molecule modulators

[0062] Small molecules, are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents,

referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight less than 10,000, preferably less than 5,000, more preferably less than 1,000, and most preferably less than 500. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the RRP protein or may be identified by screening compound libraries. Alternative appropriate modulators of this class are natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for RRP–modulating activity. Methods for generating and obtaining compounds are well known in the art (Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 151:1947-1948).

[0063] Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with the p53 or p21 pathway. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

Protein Modulators

[0064] Specific RRP-interacting proteins are useful in a variety of diagnostic and therapeutic applications related to the p53 or p21 pathway and related disorders, as well as in validation assays for other RRP-modulating agents. In a preferred embodiment, RRP-interacting proteins affect normal RRP function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In another embodiment, RRP-interacting proteins are useful in detecting and

providing information about the function of RRP proteins, as is relevant to p53 or p21 related disorders, such as cancer (e.g., for diagnostic means).

[0065] An RRP-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with an RRP, such as TGFα, EGF, amphiregulin, heregulin, a member of the RRP pathway that modulates RRP expression, localization, and/or activity. RRP-modulators include dominant negative forms of RRP-interacting proteins and of RRP proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous RRP-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3rd, Trends Genet (2000) 16:5-8).

[0066] An RRP-interacting protein may be an exogenous protein, such as an RRP-specific antibody or a T-cell antigen receptor (see, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory). RRP antibodies are further discussed below.

[0067] In preferred embodiments, an RRP-interacting protein specifically binds an RRP protein. In alternative preferred embodiments an RRP-modulating agent binds an RRP substrate, binding partner, or cofactor.

Antibodies

[0068] In another embodiment, the protein modulator is an RRP specific antibody agonist or antagonist. The antibodies have therapeutic and diagnostic utilities, and can be used in screening assays to identify RRP modulators. For example, uses for antibodies include the detection of an RRP protein in a biological sample and the inhibition of RRP activity, for instance, to block the development of an oncogenic disorder. The antibodies can also be used in dissecting the portions of the RRP

pathway responsible for various cellular responses and in the general processing and maturation of the RRP.

[0069] Antibodies that specifically bind RRP polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of RRP polypeptide, and more preferably, to human RRP. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab').sub.2 fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 M^{-1} to 10^{10} M^{-1}, or stronger can be made by standard procedures as described (Harlow and Lane, Antibodies: A Laboratory Manual, CSH Laboratory (1988); Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against crude cell extracts of RRP or substantially purified fragments thereof. If RRP fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of an RRP protein. In a particular embodiment, RRP-specific antigens and/or immunogens are coupled to carrier proteins that stimulate the immune response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier, and the conjugate is emulsified in Freund's complete adjuvant, which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols. In a preferred embodiment, due to close similarity of RRP sequences from mice and humans, transgenic mice that are RRP deficient or RRP knockout, such as those generated in the present invention (Example VIII), are used to produce antibodies against human RRP.

[0070] The presence of RRP-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding RRP polypeptides. Other assays, such as radioimmunoassays or fluorescent assays might also be used.

[0071] Chimeric antibodies specific to RRP polypeptides can be made that contain different portions from different animal species. For instance, a human

immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al., Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 10:239-265). Humanized antibodies and methods of their production are well-known in the art (U.S. Pat. No. 5,530,101; U.S. Pat. No. 5,585,089; U.S. Pat. No. 5,693,762, and U.S. Pat. No. 6,180,370).

[0072] RRP-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No. 4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA (1988) 85:5879-5883; and Ward et al., Nature (1989) 334:544-546).

[0073] Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As used herein, T-cell antigen receptors are included within the scope of antibody modulators (Harlow and Lane, 1988, *supra*).

[0074] The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells that express the targeted protein (Menard S, et al., Int J. Biol Markers

(1989) 4:131-134). A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like (U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also, recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic polypeptides may be delivered and reach their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. No. 6,086,900).

[0075] When used therapeutically in a patient, the antibodies of the subject invention are typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg –to about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion) in association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance isotonicity and chemical stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml–to about10 mg/ml. Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; WO0073469).

[0076] The selection of an appropriate antibody subclass for therapy will depend upon the nature of the tumor antigen. For example, an IgM may be preferred when the antigen is highly specific for the tumor target and rarely occurs on normal cells. However, the IgG subclass may be preferred when the tumor-associated antigen is also expressed in normal tissues, even at much lower levels. The binding of at least two IgG molecules in close proximity is required to activate complement, a serum protein that combines with antibodies to form a defense against cellular antigens. The

normal tissues that express smaller amounts of the antigen and bind fewer IgG molecules may thus incur less complement-mediated damage. Furthermore, since IgGs are smaller than IgMs, they may more readily localize to tumor tissue.

[0077] Immune responses may assist in the delivery or efficacy of an anti-tumor treatment. There is evidence that complement activation leads to an inflammatory response and macrophage activation (Uananue and Benecerraf, Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). Activated macrophages more preferentially destroy tumor cells than normal cells (Fidler and Poste, Springer Semin. Immunopathol. 5, 161 (1982)). Also, the increased vasodilation accompanying inflammation may increase the ability of anti-cancer agents, such as chemotherapeutic drugs or radiolabeled antibodies to localize in tumors. While a significant detriment of standard chemotherapy or radiation treatment is damage to healthy cells, the antigen-antibody combinations specified by this invention may circumvent many of the problems normally caused by the heterogeneity of tumor cell populations. Additionally, purified antigens (Hakomori, Ann. Rev. Immunol. (1984) 2:103) or the related anti-idiotypic antibodies (Nepom et al., Proc. Natl. Acad. Sci, (1985) 81:2864; Koprowski et al., Proc. Natl. Acad. Sci. (1984) 81:216) which recognize the hypervariance among the same epitopes in different individuals could be used to induce an active immune response in human cancer patients. Such a response includes the formation of antibodies capable of activating human complement and mediating antibody-dependent cell-mediated cytotoxicity and by such mechanisms cause tumor destruction.

Specific biotherapeutics

[0078] In a preferred embodiment, an RRP-interacting protein may have biotherapeutic applications. Biotherapeutic agents formulated in pharmaceutically acceptable carriers and dosages may be used to activate or inhibit signal transduction pathways. This modulation may be accomplished by binding a ligand, thus inhibiting the activity of the pathway; or by binding a receptor, either to inhibit activation of, or to activate, the receptor. Alternatively, the biotherapeutic may itself be a ligand capable

of activating or inhibiting a receptor. Biotherapeutic agents and methods of producing them are described in detail in U.S. Pat. No. 6,146,628.

[0079] Since RRP is a receptor, its ligand(s), antibodies to the ligand(s) or the RRP itself may be used as biotherapeutics to modulate the activity of RRP in the p53 or p21 pathway.

Nucleic Acid Modulators

[0080] Other preferred RRP-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit RRP activity.

[0081] Preferred antisense oligomers that interfere with the function of the RRP nucleic acid such as DNA replication, transcription, translocation of the RRP RNA to the site of protein translation, translation of protein from the RRP RNA, splicing of the RRP RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RRP RNA. Double-stranded RNA inhibition (dsRNAi) is another preferred RRP-modulating agent. For convenience, the term "antisense modulator", as used herein, includes antisense oligomers and dsRNAi.

[0082] In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to an RRP mRNA to bind to and prevent translation, preferably by binding to the 5' untranslated region. RRP-specific antisense oligonucleotides, preferably range from at least 6 to about 200 nucleotides. In some embodiments the oligonucleotide is preferably at least 10, 15, or 20 nucleotides in length. In other embodiments, the oligonucleotide is preferably less than 50, 40, or 30 nucleotides in length. The oligonucleotide can be DNA or RNA or a chimeric mixture or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

[0083] In another embodiment, the antisense oligomer is a phosphothioate morpholino oligomer (PMO). PMOs are assembled from four different morpholino subunits,

each of which contain one of four genetic bases (A, C, G, or T) linked to a six-membered morpholine ring. Polymers of these subunits are joined by non-ionic phosphodiamidate intersubunit linkages. Details of how to make and use PMOs and other antisense oligomers are well known in the art (e.g. see WO99/18193; Probst JC, Antisense Oligodeoxynucleotide and Ribozyme Design, Methods. (2000) 22(3):271-281; Summerton J, and Weller D. 1997 Antisense Nucleic Acid Drug Dev. ;7:187-95, US Pat. No. 5,235,033; and US Pat No. 5,378,841).

[0084] Antisense oligomers are commonly used as research reagents, diagnostics, and therapeutics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used to elucidate the function of particular genes (see, for example, U.S. Pat. No. 6,165,790). Antisense oligomers are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense oligomers have been employed as therapeutic moieties in the treatment of disease states in animals and man and have been demonstrated in numerous clinical trials to be safe and effective (Milligan JF, *et al*, Current Concepts in Antisense Drug Design, J Med Chem. (1993) 36:1923-1937; Tonkinson JL *et al.*, Antisense Oligodeoxynucleotides as Clinical Therapeutic Agents, Cancer Invest. (1996) 14:54-65). Accordingly, in one aspect of the invention, an RRP-specific antisense oligomer is used in an assay to further elucidate the role of the RRP in the p53 or p21 pathway, and/or its relationship to other members of the pathway. In another aspect of the invention, an RRP-specific antisense oligomer is used as a therapeutic agent for treatment of p53 or p21-related disease states.

[0085] Alternative preferred RRP-modulating agents are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and humans are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-245 (2001); Hamilton, A. et al., Science

286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619; Elbashir SM, et al., 2001 Nature 411:494-498).

Assay Systems

[0086] The invention provides assay systems for identifying specific modulators of RRP activity. As used herein, an "assay system" encompasses all the components required for performing and analyzing results of an assay that detects and/or measures a particular event. In general, primary assays are used to identify or confirm a modulator's specific biochemical or molecular effect with respect to the RRP nucleic acid or protein. In general, secondary assays further assess the activity of a RRP modulating agent identified by a primary assay and may confirm that the modulating agent affects RRP in a manner relevant to the p53 or p21 pathway. In some cases, RRP modulators will be directly tested in a secondary assay.

[0087] In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising an RRP polypeptide with a candidate agent under conditions whereby, but for the presence of the agent, the system provides a reference activity (e.g. protease activity), which is based on the particular molecular event the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates RRP activity, and hence the p53 or p21 pathway.

Primary Assays

[0088] The type of modulator tested generally determines the type of primary assay.

Primary assays for small molecule modulators

[0089] For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam GS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying

references). As used herein the term "cell-based" refers to assays using live cells, dead cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. The term "cell free" encompasses assays using substantially purified protein (either endogenous or recombinantly produced), partially purified cellular extracts, or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding), transcriptional activity (*e.g.*, using a reporter gene), enzymatic activity (*e.g.*, via a property of the substrate), activity of second messengers, immunogenicty and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide range of detection methods including fluorescent, radioactive, colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular molecular event detected.

[0090] In a preferred embodiment, screening assays uses fluorescence technologies, including fluorescence polarization, time-resolved fluorescence, and fluorescence resonance energy transfer. These systems offer means to monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.*, Selvin PR, Nat Struct Biol (2000) 7:730-4; Fernandes PB, Curr Opin Chem Biol (1998) 2:597-603; Hertzberg RP and Pope AJ, Curr Opin Chem Biol (2000) 4:445-451).

[0091] Cell-based screening assays usually require systems for recombinant expression of RRP and any auxiliary proteins demanded by the particular assay. Cell-free assays often use recombinantly produced purified or substantially purified proteins. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and assure assay reproducibility. Yeast two-hybrid and variant screens, and mass spectrometry provide preferred methods for determining protein-protein interactions and elucidation of protein complexes. In certain applications, when RRP-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the RRP protein may be assayed by various known methods such as substrate processing

(e.g. ability of the candidate RRP-specific binding agents to function as negative effectors in RRP-expressing cells), binding equilibrium constants (usually at least about 10^7 M^{-1}, preferably at least about 10^8 M^{-1}, more preferably at least about 10^9 M^{-1}), and immunogenicity (e.g. ability to elicit RRP specific antibody in a heterologous host such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

[0092] The screening assay may measure a candidate agent's ability to specifically bind to or modulate activity of a RRP polypeptide, a fusion protein thereof, or to cells or membranes bearing the polypeptide or fusion protein. The RRP polypeptide can be full length or a fragment thereof that retains functional RRP activity. The RRP polypeptide may be fused to another polypeptide, such as a peptide tag for detection or anchoring, or to another tag. The RRP polypeptide is preferably human or mouse RRP, or is an ortholog or derivative thereof as described above. In a preferred embodiment, the screening assay detects candidate agent-based modulation of RRP interaction with a binding target, such as an endogenous or exogenous protein or other substrate that has RRP –specific binding activity, and can be used to assess normal RRP gene function.

[0093] Suitable assay formats that may be adapted to screen for RRP modulators are known in the art. Preferred screening assays are high throughput or ultra high throughput and thus provide automated, cost-effective means of screening compound libraries for lead compounds (Fernandes PB, 1998, *supra*; Sundberg SA, Curr Opin Biotechnol 2000, 11:47-53).

[0094] A variety of suitable assay systems may be used to identify candidate RRP and p53 or p21 pathway modulators (e.g. U.S. Pat. No. 6,020,135 (p53 modulation), U.S. Pat. No. 6,114,132 (phosphatase and protease assays)). Specific preferred assays are described in more detail below.

[0095] **Protease Assays.** Proteases are enzymes that cleave protein substrates at specific sites. Exemplary assays detect the alterations in the spectral properties of an artificial substrate that occur upon protease-mediated cleavage. In one example, synthetic caspase substrates containing four amino acid proteolysis recognition sequences,

separating two different fluorescent tags are employed; fluorescence resonance energy transfer detects the proximity of these fluorophores, which indicates whether the substrate is cleaved (Mahajan NP *et al.*, Chem Biol (1999) 6:401-409).

[0096] Endogenous protease inhibitors may inhibit protease activity. In an example of an assay developed for either proteases or protease inhibitors, a biotinylated substrate is coated on a titer plate and hydrolyzed with the protease; the unhydrolyzed substrate is quantified by reaction with alkaline phosphatase-streptavidin complex and detection of the reaction product. The activity of protease inhibitors correlates with the activity of the alkaline phosophatase indicator enzyme (Gan Z *et al.*, Anal Biochem 1999) 268:151-156).

[0097] **Apoptosis assays.** Assays for apoptosis may be performed by terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL) assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, 1994, Nature 371, 346), by following the incorporation of fluorescein-dUTP (Yonehara *et al.*, 1989, J. Exp. Med. 169, 1747). Apoptosis may further be assayed by acridine orange staining of tissue culture cells (Lucas, R., et al., 1998, Blood 15:4730-41). An apoptosis assay system may comprise a cell that expresses an RRP, and that optionally has defective p53 or p21 function (e.g. p53 or p21 is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the apoptosis assay system and changes in induction of apoptosis relative to controls where no test agent is added, identify candidate p53 or p21 modulating agents. In some embodiments of the invention, an apoptosis assay may be used as a secondary assay to test a candidate p53 or p21 modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether RRP function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express RRP relative to wild type cells. Differences in apoptotic response compared to wild type cells suggests that the RRP plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

[0098] **Cell proliferation and cell cycle assays.** Cell proliferation may be assayed via bromodeoxyuridine (BRDU) incorporation. This assay identifies a cell population undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (Hoshino *et al.*, 1986, Int. J. Cancer 38, 369; Campana *et al.*, 1988, J. Immunol. Meth. 107, 79), or by other means.

[0099] Cell Proliferation may also be examined using [^{3}H]-thymidine incorporation (Chen, J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [^{3}H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation Counter).

[00100] Cell proliferation may also be assayed by colony formation in soft agar (Sambrook et al., Molecular Cloning, Cold Spring Harbor (1989)). For example, cells transformed with RRP are seeded in soft agar plates, and colonies are measured and counted after two weeks incubation.

[00101] Involvement of a gene in the cell cycle may be assayed by flow cytometry. Cells transfected with an RRP may be stained with propidium iodide and evaluated in a flow cytometer (available from Becton Dickinson).

[00102] Accordingly, a cell proliferation or cell cycle assay system may comprise a cell that expresses an RRP, and that optionally has defective p53 or p21 function (e.g. p53 or p21 is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where no test agent is added, identify candidate p53 or p21 modulating agents. In some embodiments of the invention, the cell proliferation or cell cycle assay may be used as a secondary assay to test a candidate p53 or p21 modulating agents that is initially identified using another assay system such as a cell-free kinase assay system. A cell proliferation assay may also be used to test whether RRP function plays a direct role in cell proliferation or cell cycle. For example, a cell proliferation or cell cycle assay may be performed on cells that over- or under-

express RRP relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the RRP plays a direct role in cell proliferation or cell cycle. A cell proliferation assay may also be used to identify candidate agents that modulate cell proliferation. For example, cells that have decreased expression of RRP or that are RRP knockouts, such as mouse cells generated in the present invention (Example VIII) are treated with candidate agents. Changes in cell proliferation relative to control cells where no agent is added indicate that the candidate agent modulates cell proliferation.

[00103] **Angiogenesis.** Angiogenesis may be assayed using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses an RRP, and that optionally has defective p53 or p21 function (e.g. p53 or p21 is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the angiogenesis assay system and changes in angiogenesis relative to controls where no test agent is added, identify candidate p53 or p21 modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate p53 or p21 modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether RRP function plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express RRP relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the RRP plays a direct role in angiogenesis.

[00104] **Hypoxic induction.** The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumor cell survival, such as those encoding glyolytic enzymes and VEGF. Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with RRP in hypoxic conditions (such as with 0.1% O2, 5% CO2, and balance N2, generated in a Napco 7001 incubator (Precision Scientific)) and normoxic conditions, followed by assessment of gene activity or expression by Taqman®. For example, a hypoxic induction assay system may comprise a cell that expresses an RRP, and that optionally has a mutated p53 or p21 (e.g. p53 or p21 is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate p53 or p21 modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a candidate p53 or p21 modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether RRP function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may be performed on cells that over- or under-express RRP relative to wild type cells. Differences in hypoxic response compared to wild type cells suggest that the RRP plays a direct role in hypoxic induction.

[00105] **Cell adhesion.** Cell adhesion assays measure adhesion of cells to purified adhesion proteins, or adhesion of cells to each other, in presence or absence of candidate modulating agents.

[00106] Cell-protein adhesion assays measure the ability of agents to modulate the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2× final test concentration and added to the blocked, coated wells. Cells are then added to the wells, and the unbound cells are washed off. Retained cells are labeled directly on

the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

[00107] Cell-cell adhesion assays measure the ability of agents to modulate binding of cell adhesion proteins with their native ligands. These assays use cells that naturally or recombinantly express the adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate. Cells expressing the ligand are labeled with a membrane-permeable fluorescent dye, such as BCECF , and allowed to adhere to the monolayers in the presence of candidate agents. Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader.

[00108] High-throughput cell adhesion assays have also been described. In one such assay, small molecule ligands and peptides are bound to the surface of microscope slides using a microarray spotter, intact cells are then contacted with the slides, and unbound cells are washed off. In this assay, not only the binding specificity of the peptides and modulators against cell lines are determined, but also the functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip is measured (Falsey JR et al., Bioconjug Chem. 2001 May-Jun;12(3):346-53).

[00109] Certain screening assays may also be used to test antibody and nucleic acid modulators; for nucleic acid modulators, appropriate assay systems involve RRP mRNA expression.

Primary assays for antibody modulators

[00110] For antibody modulators, appropriate primary assays test is a binding assay that tests the antibody's affinity to and specificity for the RRP protein. Methods for testing antibody affinity and specificity are well known in the art (Harlow and Lane, 1988, 1999, *supra*). The enzyme-linked immunosorbant assay (ELISA) is a preferred method for detecting RRP-specific antibodies; others include FACS assays, radioimmunoassays, and fluorescent assays.

Primary assays for nucleic acid modulators

[00111] For nucleic acid modulators, primary assays may test the ability of the nucleic acid modulator to inhibit or enhance RRP gene expression, preferably mRNA expression. In general, expression analysis comprises comparing RRP expression in like populations of cells (*e.g.*, two pools of cells that endogenously or recombinantly express RRP) in the presence and absence of the nucleic acid modulator. Methods for analyzing mRNA and protein expression are well known in the art. For instance, Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR (*e.g.*, using the TaqMan®, PE Applied Biosystems), or microarray analysis may be used to confirm that RRP mRNA expression is reduced in cells treated with the nucleic acid modulator (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, *eds.*, John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm DH and Guiseppi-Elie, A Curr Opin Biotechnol 2001, 12:41-47). Protein expression may also be monitored. Proteins are most commonly detected with specific antibodies or antisera directed against either the RRP protein or specific peptides. A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

Secondary Assays

[00112] Secondary assays may be used to further assess the activity of RRP-modulating agent identified by any of the above methods to confirm that the modulating agent affects RRP in a manner relevant to the p53 or p21 pathway. As used herein, RRP-modulating agents encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulator on a particular genetic or biochemical pathway or to test the specificity of the modulator's interaction with RRP.

[00113] Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express RRP) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate RRP–modulating agent results

in changes in the p53 or p21 pathway in comparison to untreated (or mock- or placebo-treated) cells or animals. Certain assays use "sensitized genetic backgrounds", which, as used herein, describe cells or animals engineered for altered expression of genes in the p53 or p21 or interacting pathways.

Cell-based assays

[00114] Cell based assays may use a variety of mammalian cell lines known to have defective p53 or p21 function (e.g. for p53: SAOS-2 osteoblasts, H1299 lung cancer cells, C33A and HT3 cervical cancer cells, HT-29 and DLD-1 colon cancer cells, among others, and for p21: HCT116 colon cancer cells, among others, available from American Type Culture Collection (ATCC), Manassas, VA). Cell based assays may detect endogenous p53 or p21 pathway activity or may rely on recombinant expression of p53 or p21 pathway components. Any of the aforementioned assays may be used in this cell-based format. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

Animal Assays

[00115] A variety of non-human animal models of normal or defective p53 or p21 pathway may be used to test candidate RRP modulators. Models for defective p53 or p21 pathway typically use genetically modified animals that have been engineered to mis-express (*e.g.*, over-express or lack expression in) genes involved in the p53 or p21 pathway. Assays generally require systemic delivery of the candidate modulators, such as by oral administration, injection, etc.

[00116] In a preferred embodiment, p53 or p21 pathway activity is assessed by monitoring neovascularization and angiogenesis. Animal models with defective and normal p53 or p21 are used to test the candidate modulator's affect on RRP in Matrigel® assays. Matrigel® is an extract of basement membrane proteins, and is composed primarily of laminin, collagen IV, and heparin sulfate proteoglycan. It is provided as a sterile liquid at 4° C, but rapidly forms a solid gel at 37° C. Liquid Matrigel® is mixed with various angiogenic agents, such as bFGF and VEGF, or with human tumor cells

which over-express the RRP. The mixture is then injected subcutaneously into the female athymic nude mice (Taconic, Germantown, NY) to support an intense vascular response. Mice with Matrigel® pellets may be dosed via oral (PO), intraperitoneal (IP), or intravenous (IV) routes with the candidate modulator. Mice are euthanized 5 - 12 days post-injection, and the Matrigel® pellet is harvested for hemoglobin analysis (Sigma plasma hemoglobin kit). Hemoglobin content of the gel is found to correlate the degree of neovascularization in the gel.

[00117] In another preferred embodiment, the effect of the candidate modulator on RRP is assessed via tumorigenicity assays. In one example, xenograft human tumors are implanted subcutaneously (SC) into female athumic nude mice, 6-7 week old, as single cell suspensions either from a pre-existing tumor or from *in vitro* culture. The tumors which express the RRP endogenously are injected in the flank, 1 x 10^5 to 1 x 10^7 cells per mouse in a volume of 100 μL using a 27gauge needle. Mice are then ear tagged and tumors are measured twice weekly. Candidate modulator treatment is initiated on the day the mean tumor weight reaches 100 mg. Candidate modulator is delivered intravenously (IV), subcutaneously (SC), intraperitoneously (IP), or orally (PO) by bolus administration. Depending upon the pharmacokinetics (PK) of each unique candidate modulator, dosing can be performed multiple times per day. The tumor weight is assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. At the end of the experiment, the excised tumors maybe utilized for biomarker identification or further analyses. For immunohischemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, PH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopetane cooled with liquid nitrogen.

Diagnostic and therapeutic uses

[00118] Specific RRP-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in the p53 or p21 pathway, such as angiogenic, apoptotic, or cell proliferation disorders. Accordingly, the invention also provides methods for modulating the p53 or p21 pathway in a cell, preferably a cell pre-determined to have defective p53 or p21

function, comprising the step of administering an agent to the cell that specifically modulates RRP activity.

[00119] The discovery that RRP is implicated in p53 or p21 pathway provides for a variety of methods that can be employed for the diagnostic and prognostic evaluation of diseases and disorders involving defects in the p53 or p21 pathway and for the identification of subjects having a predisposition to such diseases and disorders.

[00120] Various expression analysis methods can be used to diagnose whether RRP expression occurs in a particular sample, including Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR, and microarray analysis. (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, *eds.*, John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm and Guiseppi-Elie, Curr Opin Biotechnol 2001, 12:41-47). Tissues having a disease or disorder implicating defective p53 or p21 signaling that express an RRP, are identified as amenable to treatment with an RRP modulating agent. In a preferred application, the p53 or p21 defective tissue overexpresses an RRP relative to normal tissue. For example, a Northern blot analysis of mRNA from tumor and normal cell lines, or from tumor and matching normal tissue samples from the same patient, using full or partial RRP cDNA sequences as probes, can determine whether particular tumors express or overexpress RRP. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of RRP expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

[00121] Various other diagnostic methods may be performed, for example, utilizing reagents such as the RRP oligonucleotides, and antibodies directed against an RRP, as described above for: (1) the detection of the presence of RRP gene mutations, or the detection of either over- or under-expression of RRP mRNA relative to the non-disorder state; (2) the detection of either an over- or an under-abundance of RRP gene product relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by RRP.

[00122] Thus, in a specific embodiment, the invention is drawn to a method for diagnosing a disease in a patient, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for RRP expression;

c) comparing results from step (b) with a control; and d) determining whether step (c) indicates a likelihood of disease. Preferably, the disease is cancer, most preferably a cancer as shown in TABLE 1. The probe may be either DNA or protein, including an antibody.

EXAMPLES

[00123] The following experimental section and examples are offered by way of illustration and not by way of limitation.

I. Drosophila p53 and p21 screens

[00124] The Drosophila p53 gene was overexpressed specifically in the wing using the vestigial margin quadrant enhancer. Increasing quantities of Drosophila p53 (titrated using different strength transgenic inserts in 1 or 2 copies) caused deterioration of normal wing morphology from mild to strong, with phenotypes including disruption of pattern and polarity of wing hairs, shortening and thickening of wing veins, progressive crumpling of the wing and appearance of dark "death" inclusions in wing blade. In a screen designed to identify enhancers and suppressors of Drosophila p53, homozygous females carrying two copies of p53 were crossed to 5663 males carrying random insertions of a piggyBac transposon (Fraser M *et al.*, Virology (1985) 145:356-361). Progeny containing insertions were compared to non-insertion-bearing sibling progeny for enhancement or suppression of the p53 phenotypes. Sequence information surrounding the piggyBac insertion site was used to identify the modifier genes. Modifiers of the wing phenotype were identified as members of the p53 pathway.

[00125] An overexpression screen was carried out in *Drosophila* to identify genes that interact with the cyclin dependent kinase inhibitor, p21 (Bourne HR, et al., Nature (1990) 348(6297):125-132; Marshall CJ, Trends Genet (1991) 7(3):91-95). Expression of the p21 gene in the eye causes deterioration of normal eye morphology. Modifiers of the eye phenotype were identified as members of the p21 pathway.

II. High-Throughput In Vitro Fluorescence Polarization Assay

[00126] Fluorescently-labeled RRP peptide/substrate are added to each well of a 96-well microtiter plate, along with a test agent in a test buffer (10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6). Changes in fluorescence polarization, determined by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc), relative to control values indicates the test compound is a candidate modifier of RRP activity.

III. High-Throughput In Vitro Binding Assay.

[00127] ^{33}P-labeled RRP peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM $MgCl_2$, 1% glycerol, 0.5% NP-40, 50 mM beta-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors) along with a test agent to the wells of a Neutralite-avidin coated assay plate and incubated at 25°C for 1 hour. Biotinylated substrate is then added to each well and incubated for 1 hour. Reactions are stopped by washing with PBS, and counted in a scintillation counter. Test agents that cause a difference in activity relative to control without test agent are identified as candidate p53 or p21 modulating agents.

IV. Immunoprecipitations and Immunoblotting

[00128] For coprecipitation of transfected proteins, 3×10^6 appropriate recombinant cells containing the RRP proteins are plated on 10-cm dishes and transfected on the following day with expression constructs. The total amount of DNA is kept constant in each transfection by adding empty vector. After 24 h, cells are collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM -glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris is removed by centrifugation twice at 15,000 × g for 15 min. The cell lysate is incubated with 25 μl of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.

[00129] After extensive washing with lysis buffer, proteins bound to the beads are solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with the indicated antibodies. The reactive bands are visualized with horseradish peroxidase coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

V. Expression analysis

[00130] All cell lines used in the following experiments are NCI (National Cancer Institute) lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues were obtained from Impath, UC Davis, Clontech, Stratagene, and Ambion.

[00131] TaqMan analysis was used to assess expression levels of the disclosed genes in various samples.

[00132] RNA was extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/μl. Single stranded cDNA was then synthesized by reverse transcribing the RNA samples using random hexamers and 500ng of total RNA per reaction, following protocol 4304965 of Applied Biosystems (Foster City, CA, http://www.appliedbiosystems.com/).

[00133] Primers for expression analysis using TaqMan assay (Applied Biosystems, Foster City, CA) were prepared according to the TaqMan protocols, and the following criteria: a) primer pairs were designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product.

[00134] Taqman reactions were carried out following manufacturer's protocols, in 25 μl total volume for 96-well plates and 10 μl total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis was prepared using a universal pool of human cDNA samples, which is a mixture of cDNAs from a wide variety of tissues so that the

chance that a target will be present in appreciable amounts is good. The raw data were normalized using 18S rRNA (universally expressed in all tissues and cells).

[00135] For each expression analysis, tumor tissue samples were compared with matched normal tissues from the same patient. A gene was considered overexpressed in a tumor when the level of expression of the gene was 2 fold or higher in the tumor compared with its matched normal sample. In cases where normal tissue was not available, a universal pool of cDNA samples was used instead. In these cases, a gene was considered overexpressed in a tumor sample when the difference of expression levels between a tumor sample and the average of all normal samples from the same tissue type was greater than 2 times the standard deviation of all normal samples (i.e., Tumor – average(all normal samples) > 2 x STDEV(all normal samples)).

[00136] Results are shown in Table 1. Data presented in bold indicate that greater than 50% of tested tumor samples of the tissue type indicated in row 1 exhibited over expression of the gene listed in column 1, relative to normal samples. Underlined data indicates that between 25% to 49% of tested tumor samples exhibited over expression. A modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a patient with the modulator, the likelihood that the patient will respond to treatment can be diagnosed by obtaining a tumor sample from the patient, and assaying for expression of the gene targeted by the modulator. The expression data for the gene(s) can also be used as a diagnostic marker for disease progression. The assay can be performed by expression analysis as described above, by antibody directed to the gene target, or by any other available detection method.

TABLE I

Gene Name	Breast Matched	Breast Total	Colon Matched	Lung Matched	Ovary Matched	Ovary Total	Kidney Matched
RRP1	0 / 3	0 / 5	11 / 26	**8 / 14**	1 / 4	3 / 14	2 / 19
RRP2	1 / 3	1 / 5	3 / 26	**7 / 14**	**2 / 4**	**9 / 14**	**12 / 19**
RRP3	0 / 3	0 / 5	4 / 26	4 / 14	1 / 4	3 / 14	4 / 19
RRP4	0 / 3	0 / 5	3 / 26	3 / 14	1 / 4	5 / 14	4 / 19
RRP5	0 / 3	0 / 5	6 / 26	1 / 14	1 / 4	1 / 14	4 / 19
RRP6	**2 / 3**	**3 / 5**	10 / 26	5 / 14	0 / 4	0 / 14	**13 / 19**
RRP7	0 / 3	0 / 5	6 / 26	1 / 14	1 / 4	1 / 14	1 / 19
RRP8	1 / 3	1 / 5	6 /26	5 / 14	0 / 4	0 / 14	3 / 19

VI. Cell Biology and Functional Characterization

[00137] RRP Localization. In Drosophila, rhomboid is a cell surface protein. However, localization of human RRPs has not happened to date. Furthermore, in humans, it is not known whether each of the termini of the rhomboids is intracellular or extracellular. To answer these questions, RRP1 was subcloned into pcDNA expression vector (Invitrogen) in frame with myc-his tag at the c-terminus, according to manufacturer's protocols. The resulting expression vector was transiently transfected using Lipofectamine Plus reagent (Life technologies) into human embryonic kidney HEK-293 cells. Immunofluorescence staining using anti-myc antibody was then carried out on the cells to localize RRP1. Results of these experiments indicated that RRP1 is expressed at the cell surface. Furthermore, permeabilized (0.1% triton in PBS) and non-permeabilized cells show the same staining pattern, indicating that the c-terminus of the protein is outside, and the N-terminus, inside. RRP2 – RRP8 are also subcloned and tagged at the c-terminus for localization of each protein, and also to assess the direction of the protein ends in each case.

[00138] **Involvement of RRP in EGFR signaling pathway.** While Drosophila rhomboid is essential in the EGFR pathway, the function of human RRPs have not been assessed to date. To assess the role of human RRPs in the EGFR signaling pathway, RRP1 was stably transfected using CaPO4 transfection kit (Clontech) into Hela cells, which have endogenous EGFR activity. Pooled stable cells were then examined in the following assays.

EGFR activation as measured by tyrosine phosphorylation

[00139] Hela cells overexpressing RRP1 and parental Hela cells were tested for EGFR tyrosine phosphorylation by immunoblot against anti-phosphotyrosine using anti-phosphotyrosine antibody (Upstate Biotechnology). Cells overexpressing RRP1 demonstrated up to two-fold increase in EGFR tyrosine phosphorylation as compared to parental Hela controls.

EGFR expression in cells overexpressing RRP1.

[00140] Expression of EGFR was increased when tested by immunoblot using anti-EGFR antibody against direct cell lysates in cells overexpressing RRP1. Furthermore, expression of EGFR was also increased in tumor samples overexpressing RRP1, by TaqMan® analysis. The correlation of both in-vitro and in-vivo results for concordant overexpression of EGFR and RRP1 is quite significant, and provides a tool for assessing the functional relationship of the two proteins in any tumor sample or cell line.

Cell proliferation and Migration.

[00141] Hela cells overexpressing RRP1 demonstrate a two-fold increase in cell proliferation compared to Hela parental cells as measured by Procheck (Intergen) and AlamarBlue (Biosource International) chemiluminescent assays. In addition, Fluroblok (BD Biosource) analysis showed a slight increase in motility in cells overexpressing RRP1 compared to Hela parental cells.

[00142] Taken together, these results demonstrate a strong functional conservation of rhomboids across evolution. Furthermore, increased RRP1 expression in tumor samples and cell lines, concurrent increased expression of EGFR, and increased proliferation and motility of cells expressing RRP1 suggest involvement of RRP1 in various cancers. RRP1 expression levels can thus be used to screen for tumors with

defective EGFR pathways, to identify tumors amenable to treatment with the compounds and antibodies identified by the methods of the present invention. Same aforementioned experiments are performed with RRP2 – RRP8 to assess their involvement in the EGFR signaling pathway.

[00143] **RRP1 binding target**. In Drosophila, rhomboid transforms Spitz (homolog of human TGF-α) from a membrane-bound to a secreted form, and thus triggers and upregulates the DER (Drosophila EGFR) pathway. No such function has been shown for human rhomboids. HEK293 cells were transiently co-transfected with RRP1 and TM-TGF- α transmembrane-bound TGF), then immunoprecipitated with anti-myc antibody (for RRP1) and immunoblotted with anti-TGF-α. Cells transfected with vector alone, RRP1 alone, or TM-TGF-α alone were used as controls. A band corresponding to the size of TGF- α was recognized by anti-TGF- α antibody, while no bands were detected in the control lanes. This data indicates that RRP1 physically interacts with TGF- α. The same experiments are performed with RRP2 – RRP8 to determine their binding partners.

[00144] **Antibodies against human rhomboids.** A peptide antibody, SEQ ID NO: 17 , and referred to as peptide 1, was made against the N-terminus of RRP1. This antibody displayed strong affinity for denatured RRP1, such as in cells fixed with formalin or proteins on western blots. In addition, staining pattern remained the same when permeabilized cells were co-stained with anti-myc antibodies (for the c-terminus of RRP1) and peptide 1, suggesting that Peptide 1 does recognize RRP1. Furthermore, the cells were significantly immunostained only when they were permeabilized, suggesting that the N-terminus of RRP1 is inside the cells. This result correlates with the previous staining pattern using anti-myc antibody for the C-terminus.

[00145] RRP1 protein expression in tumors was examined by Immunohistochemistry (IHC) using peptide 1. Positive staining on epithelial cells of colon adenocarcinoma tissue sections were observed as compared to background staining in preimmune serum, suggesting the antibody is specific for its target, RRP1, in colon cancer cells.

Moreover, increased staining of colorectal adenocarcinomas was observed compared to matched normal tissues using peptide 1 antibody. Taken together, these results suggest that peptide 1 antibody is a powerful tool to assess expression levels of RRP1 in tumor samples. Antibodies are also produced against the rest of RRPs to assess their expression levels in tumors.

VII. Mouse RRP1 (mRRP1) gene structure

[00146] A BAC shotgun sequencing approach (for a review, see Green ED. (2001) Nature Review Genetics 2:573-583) was used to determine the intron-exon structure of the murine RRP1 gene. Based upon the genebank entries of a partial rat cDNA and the human gene structure, a PCR primer pair was designed that allowed the amplification of a part of the murine RRP1 gene from mouse genomic DNA. (RRP1s (SEQ ID NO:48): 5´-CACCCACCAGCCGCACTGGTC; RRP8as (SEQ ID NO:49): 5´-GAGCAGCTAGGGTTCAATG, 95°C 120sec, 95°C 45sec, 60°C 45sec, 72°C 45sec, 40cycles, 1.5mM MgCl2). This PCR primer set was then used to screen a commercially available ES-129SvJ BAC library from Incyte Genomics (St. Louis, USA). 2 PCR positive clones were identified (clone addresses 178/O15 and 195/D15). Clone 195/D15 was chosen to construct a shotgun sequencing library according to standard procedures (TOPO Shotgun Subcloning Kit, #K-7000-01, Invitrogen). Upon sequence assembly, a contiguous 30 Kilobases (kb) genomic sequence was identified that carried the entire mRRP1 gene. Subsequently, the DNA from clone 195/D15 BAC served as a PCR template to amplify mRRP1 genomic regions used for the gene targeting approach. The mRRP1 gene contains 8 exons, which span a genomic region of at least 2.6 kb. The translation start on the sequence stretch defined as exon 1 has not been identified so that additional 5´ exons may exist.

VIII. Generation of the mice

Targeting Strategy

[00147] We utilized a targeting strategy that allows sequential deletion of vector sequences in the same cells. Chimeras generated by injection of mRRP1 targeted ES-cells can be used to create i) KO mice harboring a completely inactivated mRRP gene

by breeding to Cre-deleter mice (described below) as well as ii) mice with functional mRRP1 that can be deleted in a tissue specific or time specific manner by breeding to FLP-deleter mice (described below).

[00148] This strategy has the advantage that the NEO selection cassette is removed in both chimeras, thus avoiding potential hypomorphic effect of the selection marker.

Targeting vector

[00149] The targeting vector, comprising the nucleotide sequence to be incorporated into the wild –type (WT) genomic sequence, and one or more selectable markers (FIG. 1) was made based on a BAC genomic clone from the mouse strain 129SvJ background obtained from Incyte Genomics. The mRRP1 targeting vector was designed such that exons 2-8 of the mRRP1 gene were flanked with Cre recombinase recognition sequences (loxP sites) after homologous recombination. Exons 2-8 of the murine RRP encode the rhomboid domain which provide protein activity, therefore a deletion of exons 2-8 results in a null allele. Three genomic fragments were amplified by PCR and cloned individually in reversed orientation into the base vector pEasyfloxFRTGK12 (FIG. 1). This base vector has: an ampicillin resistance gene; an origin of replication; a pgtN29 plasmid (New England Biolabs), used as the source of pgk neo; pGT60mcs plasmid (InvivoGen), used as the source for TK reading frame; and LoxP and FRT sites, introduced by synthetic oligonucleotides. The 5′ region of homology (fragment A) amplified with oligo 164 (SEQ ID NO:62) and oligo 144 (SEQ ID NO:50) covered a 3.5 kb fragment that was inserted into PacI / PmeI sites upstream the 2nd loxP site of pEasyfloxFRTGK12. A 2.5 kb 3′ fragment encompassing exons 2-8 (fragment B) and amplified with oligos 145 (SEQ ID NO:51) and 146 (SEQ ID NO:52) was cloned into a AscI / FseI I sites downstream of 2nd loxP site and upstream of 2nd FRT site and *PGK-neo* cassette of pEasyfloxFRTGK12. An additional 1.0 kb fragment further 3′ (fragment C) and amplified with oligos 147 (SEQ ID NO:53) and 148 (SEQ ID NO:54) was inserted into NotI/SgfI sites located downstream the 1st FRT and 1st loxP site of pEasyfloxFRTGK12. The loxP- and FRT-flanked neomycin resistance cassette downstream of exons 2-8 of the RRP gene allowed genomic selection for homologous

recombination and a *PGK-thymidine kinase* gene cassette (PGK-TK) inserted at the 5′ end of the construct was used to select against random integration events.

[00150] In order to establish a positive control for the PCR screen on ES cell clones showing homologous recombination events, an additional 1.25 kb fragment was amplified using oligo 147 (SEQ ID NO:53) and oligo 149 (SEQ ID NO:55) and cloned into NotI / SgfI sites in pEasyfloxFRTGK12. This fragment extended the 3′ homology region of the final targeting vector by 250 base pairs, allowing establishment of PCR screening with a vector specific oligo 174 (SEQ ID NO:64) and RRP gene specific oligo 169 (SEQ ID NO:63) located 3' external in relation to the targeting vector.

Generation of targeted ES-cells

[00151] Three parental embryonic stem (ES) cell lines **B6-2** (EUROGENETEC, Ref: TG-ES01-02, derived from mouse strain C57BL/6 N TacfBr), **SvEv** (EUROGENETEC, Ref: TG-ES01-01, derived from mouse strain 129S6/SvEvTacfBr), and **E-14** ((Hooper et al., (1987) *Nature (London)* 326, 292-295); derived from mouse strain 129P2/OlaHsd) were employed for targeting of the mRRP1 locus.

[00152] All ES cell lines were cultured and electroporated under the conditions described in Torres, R. M. & Kuehn, R. Laboratory Protocols for Conditional Gene Targeting, *Oxford, New York, Tokyo:* 1997. In brief, for electroporation 1 x 10^7 cells were mixed with 30 μg of *SfiI* linearized targeting DNA in a total volume of 800 μl transfection buffer and electroporated using a Bio-Rad Gene Pulser (0,24 kV, 500 μF, 0,4 cm electrode distance). Cells were reseeded on 10-cm tissue culture dishes, containing selection resistant mouse embryonic fibroblast feeder cells (prepared from targeted mouse strain IL4 pepneo; Mueller et al., (1992) *Eur. J. Immunol.* 21: 921 – 25) at a density of 2.5 x 10^6 cells per plate. Drug selection (G418 (Gibco/BRL # 10131-019)), 200 mg/ml) was started after 1 day. ES cell clones were negatively selected the 3rd day after electroporation with the antibiotic Gancyclovir (Cymeven, Roche), 2 μM). After 8 days, resistant colonies were randomly picked and individually expanded. Correctly targeted ES clones were obtained for all 3 ES cell lines employed in the experiment.

[00153] Successful targeting of the mRRP gene was detected in resistant ES clones by PCR-screening with primer oligo 174 (SEQ ID NO:64) and oligo 169 (SEQ ID NO:63). Corecombination of the distal loxP site was detected by using oligo 200 (SEQ ID NO:65) and oligo 201 (SEQ ID NO:66). PCR-positive clones were expanded and confirmed by EcoRI / SfiI restriction enzyme digestion and Southern hybridization employing the 5' external probe amplified with oligos 152 (SEQ ID NO:56) and 153 (SEQ ID NO:57). Upon homologous recombination, the wild type 10.0 kb band was reduced to 7.5 kb (Figure 2). Corecombination of the 2nd loxP site in homologous recombinants was confirmed by HindIII and 3´ external probe amplified with oligos 160 (SEQ ID NO:60) and 162 (SEQ ID NO:61)(Figure 2). Upon corecombination of the 2nd loxP site, the wildtype 4.3 kb band was increased to 5.8 kb (Figure 2). Single integration of the targeting vector was confirmed by HindIII digestion and Southern hybridization using the internal probe amplified with oligos 154 (SEQ ID NO:58) and 155 (SEQ ID NO:59)(Figure 2).

[00154] Table 2 provides a summary of these experiments.

Table 2:Homologous recombination of the RRP-1 targeting construct in various ES cells

ES Cell	strain	# cells x 10^7	G418 res. Clones	anal.	HR	2. loxP #	Clone
B6-2	C57BL/6	4	2000	876	3	2	**G-D5** E-D9
SvEv	129S6	2	**Not counted**	95	1	0	
		3	**Not counted**	295	1	1	**D-E7**
E-14	129P2	6	**Not counted**	641	11	3	B-E2 E-F2 **C-C8**

Generation of mRRP1 chimeras

[00155] Chimeras were generated by injection of targeted ES cells into Balb/C host embryos (Hogan, B., Beddington, R., Costantini, F. & Lacy, E. eds. Manipulating the

Mouse Embryo, a Laboratory Manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor, Laboratory Press, 1994). Germline transmission was obtained by breeding chimeric mice to C57BL/6 females, resulting in mRRP1 heterozygous animals on a pure C57BL/6 strain background and hybrid (C57B/L6 x 129SvEv)$_{F1}$ and (C57B/L6 x 129P2/Ola)$_{F1}$ background.

[00156] Resulting animals were typed by Southern blot hybridization on tail DNA after digestion with HindIII with the 3′ external probe. In heterozygote animals for the target allele, the 4.3kb wild type band was increased to 5.8 kb.

[00157] Table 3 provides a summary of these experiments.

Table 3: Summary of chimera production and germline transmission of the target

Clone	blastocysts transf.	pups born	# of chimeras	GL transmis.	heterozyg. mice
B6-2					
G-D5	246	37	32	10/11	yes
E-D9	372	103	70	6/10	yes
Total	*618*	*140*	*102*	*17/21*	
SvEv					
D-E7	302	71	40	8/10	yes
E-14					
B-E2	64	17	14	8/10	yes
Grand Total	*984*	*228*	*156*	*33 / 41*	

Heterozygous animals by in vivo deletion.

Target allele with Cre directed deletion

[00158] An in vivo deletion approach was pursued to generate C57BL/6 mice carrying the target alleles with Cre-directed deletion from mRRP1. Using this approach, the Cre transgene directs the removal of the sequences flanked by the LoxP sites, and thus, creates an RRP1 knockout in every cell of the animal (FIG.3).

[00159] Heterozygote mice carrying the mRRP1 target allele (mRRP1$^{targ/+}$) from the B6-2 clones **G-D5** and **E-D9** (Table 2) were bred with transgenic C57BL/6-Cre-Deleter mice (Schwenk et al, NAR (95) Vol. 23, No 24, 5080-5081). This deleter mice have been crossed back to B6 for 10 generations, to establish the transgene on an congenic C57BL/6 background. This was done to maintain the C57BL/6 background after the

in vivo deletion of the mRRP1 targeted allele. Resulting pups were genotyped on tail DNA for the presence of the target allele with Cre-directed deletion of mRRP1 by Southern Blot hybridization and by PCR for the presence of the Cre transgene.

[00160] Tail DNA was digested with HindIII and hybridized with the 3′ external probe. In heterozygote animals the 5.8 kb band was reduced to a 1.3 kb band for the target allele. Alternatively animals were typed by PCR.

Target allele with Flp-directed deletion

[00161] An in vivo deletion approach was pursued, to generate C57BL/6 mice carrying the target allele with Flp directed deletion of the Neo marker from RRP1. In this case, the mRRP1 gene is still functional in every cell, but the animals may later be crossed with conditional Cre deleter mice for tissue specific or time-specific deletion of the sequences flanked by LoxP to create conditional mRRP1 knockout mice.

[00162] To avoid any potential hypomorphic effects, the Frt flanked Neo selection marker was removed in vivo by Flp recombination. Heterozygote mice carrying the mRRP1 target allele from the B6-2 clones **G-D5** and **E-D9** (Table 2) were bred with transgenic C57BL/6 mice heterozygous for the CAAGS-FLPe transgene (Rodriguez et al., (2000) Nat Genet 25:139-140). Resulting pups were genotyped on tail DNA for the presence of the target allele by Southern Blot hybridization and by PCR for the presence of the CAAGS-Flp transgene.

[00163] Tail DNA was digested with HindIII and hybridized the 3′ external probe. In heterozygote animals the 4.3 kb target allele band was reduced to a 3.8 kb band after the removal of the Frt-flanked Neo marker (FIG.4). Alternatively animals were typed by PCR.

Homozygous animals

Target allele with Cre directed deletion

[00164] To generate mice homozygous for the mRRP1 knockout allele on the C57BL/6 background, heterozygous animals derived from the in vivo deleted clones **G-D5** and **E-D9** were intercrossed. From this cross 17 litters were born, giving rise to 106 pups. 28 of these pups died pre-weaning. Two of these pups were recovered and genotyped

by PCR as homozygous complete KO. 49 of the living animals were weaned and 14 animals were genotyped on tail DNA with the 3′ external probe after digestion with HindIII. In homozygous animals the original 4.3 kb wild type band was reduced to a 1.3 kb band. Alternatively animals were typed by PCR.

[00165] From the 14 animals 3 typed as homozygous mutant, 7 typed as heterozygous mutant and 4 typed as wildtype. These numbers matched the expected Mendelian distribution. The homozygous animals appear to be normal. No gross difference was detected in comparison to their wildtype littermates.

[00166] To test for the fertility of the homozygous mutant animals and to increase further the colony, homozygous mutant animals for the mRRP1 derived from the in vivo deleted clones **G-D5** were intercrossed. To date, 14 pups have been born from these intercrosses, and 4 have been weaned, demonstrating the fertility of the homozygous mRRP1 KO mice.

[00167] The homozygous mRRP1 KO mice and cells are used to analyze mRRP1 function in mammals. Data from Example VI show that overexpression of human RRP1 in HeLa cells results in enhanced phosphorylation of EGFR. Inactivation of mRRP1 gene function should therefore result in attenuated phosphorylation of EGFR and or MAPK in tissues obtained from mRRP1 KO mice.

[00168] Growth factors like EGF, PDGF cause proliferation in fibroblast cell culture. As Drosophila Rhomboid and mammalian RRPs are thought to release membrane-bound growth factors by cleavage, RRP function with regard to cell cycle progression and proliferation is analyzed with embryonic and adult fibroblasts isolated from mRRP1 KO mice.

[00169] Direct effects of RRP function on tumor development is analyzed by crossing mRRP1 KO mice with APCMin mice, a model for human colon cancer. As the mRRP1 KO strain and the APCmin (Moser AR et al., (1993) Proc Natl Acad Sci U S A 90:8977-81) strain are both on C57BL/6 background, tumor formation can be analyzed on pure genetic backgrounds, and thus side effects resulting from mixed genetic backgrounds can be excluded. A strong reduction of small intestinal tumor formation in mRRP1 -/-, APCMin +/- mice versus APCMin +/- mice is expected, demonstrating that mRRP1 is a powerful target for anti-cancer drug development.

[00170] RRP is located at the cell surface and therefore a target for antibody therapy. As the extracellular loops of mouse and human RRP protein are strongly conserved, it is difficult to generate antibodies against human RRP in mice, as mice will recognize the human RRP as self. Therefore, RRP KO mice are used for the production of monoclonal antibodies directed against human RRP protein.